

Effect of ambrex (a herbal formulation) on oxidative stress in hyperlipidemic rats and differentiation of 3T3-L1 preadipocytes

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ABSTRACT

Background: Ambrex is a polyherbal formulation which consists of *Withania somnifera*, *Orchis mascula*, *Cycas circinalis*, *Shorea robusta* with amber. **Objective:** The present study was designed to explore the potential effects of ambrex on the antioxidant status in high fat diet fed rats and to investigate the possible mechanisms focusing on the gene expression involved in adipogenesis and inflammation in 3T3-L1 cell line. **Materials and Methods:** Male Wistar rats were divided into four groups ($n = 6$); Group A received normal diet, Group B received high fat diet for 30 days, Group C and D received high fat diet for 30 days and treated with ambrex (40 mg/kg b.w) and atorvastatin (10 mg/kg b.w) for successive 15 days respectively. This study also assesses the effect of ambrex on adipogenesis in 3T3-L1 adipocytes. **Results:** The serum total cholesterol and triglycerides were significantly decreased in ambrex treated hyperlipidemic animals when compared to untreated animals. The activities of catalase, superoxide dismutase and reduced glutathione were significantly augmented in the serum, liver, and heart of hyperlipidemic rats treated with ambrex when compared to control. Ambrex treated rats had significant reductions in malondialdehyde levels in the serum, liver and heart compared to untreated rats. In addition, we observed that treatment with ambrex resulted in a major inhibition of pre-adipocyte differentiation of 3T3-L1 cells *in vitro* by suppression of peroxisome proliferator activated receptor gamma, sterol regulatory binding proteins, tumor necrosis factor- α , inducible nitricoxide synthase, leptin, and upregulation of thioredoxin 1 (TRX1) and TRX2 mRNA expression. **Conclusion:** Therefore, ambrex may be a potential drug for treatment of hyperlipidemia and related disorders.

Key words: 3T3-L1 cell line, antioxidants, oxidative stress, peroxisome proliferator activated receptor gamma, sterol regulatory binding proteins

INTRODUCTION

Hyperlipidemia and related metabolic disorders are major health concerns all over the world. Oxidative stress is recently evaluated as a mechanism beneath hypercholesterolemia. Free radicals are generated as normal metabolic products of aerobic processes. Oxidative stress is an outcome of imbalance between augmented free radical production and decreased antioxidant enzyme activities. Under pathological conditions such as hypertension, hypercholesterolemia and diabetes, oxidative stress plays an important role in generating free radicals.^[1,2] Several studies report that

disease conditions are directly or indirectly associated with oxidative stress.^[3,4] Antioxidants play an important role in preventing cellular damage produced by free radicals. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione reductase-peroxidase (GSH- Px) are involved in the defensive mechanisms against oxidative stress. It is evident that hypercholesterolemia increases oxidative stress and consequently elevates lipid peroxides.^[5] Many experimental reports has determined the antioxidant enzyme activities to assess the status of free radical damage or oxidative stress.^[6-9] Therefore, antioxidant enzymes and lipid peroxidation status were assessed in serum, liver and heart of rats to determine the effect of ambrex treatment on diet induced hyperlipidemia.

We also examined the hypolipidemic potential of ambrex by determining pre-adipocyte differentiation into

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adipocytes in 3T3-L1 cell line by Oil-O red staining and peroxisome proliferator activated receptor gamma (PPAR γ), sterol regulatory binding proteins (SREBPs), leptin, tumor necrosis factor- α (TNF- α), inducible nitric oxide synthase (iNOS), thioredoxin 1 and thioredoxin 2 (TRX1), and (TRX2) gene expression analyzes. Adipose tissue is considered as a major reservoir of excess energy in the form of triglycerides whenever there is a nutritional excess. Adipocyte differentiation is well explicated by changes in the expression of various genes and transcriptional factors including nuclear hormone receptor, PPAR γ , CCAAT-enhancer binding protein and SREBPs, TNF- α , iNOS etc.

In this study, we investigated the expression of lipid metabolism regulatory genes (PPAR γ , SREBP), inflammatory related gene (TNF- α , iNOS) and oxidative stress related gene (TRX1, TRX2) to evaluate the molecular mechanisms beneath the critical factors associated with hyperlipidemia, which may detail the exact strategy of better therapy.

It is a well-known fact that PPAR γ is the prime regulator of adipogenesis. Vidal-Puig *et al.* has reported that high fat diet enhances PPAR γ expression in rodents,^[10] and humans.^[11] SREBPs transcriptionally promote the activation of an array of enzymes essential for endogenous cholesterol, fatty acids, triglycerides and phospholipid synthesis. Hence, SREBPs are the key regulators of lipid metabolism. Leptin and TNF- α are adipocytokines, which would definitely influence the adipocyte differentiation. Down-regulation of these gene expressions cause a positive impact on the control of adipogenesis. iNOS derived NO production is directly proportional to body fat composition. TRX1 and TRX2 is a ubiquitous redox protein which determines the cell proliferation of adipose tissue. As several genes are involved in maintenance of lipid homeostasis, we have analyzed a cluster of genes for better understanding of clinical manifestation associated with hyperlipidemia in *in vitro* model.

Ambrex is a polyherbal formulation, which consists of *Withania somnifera* (100 mg), *Orchis mascula* (25 mg), *Cycas circinalis* (62.5 mg), *Shorea robusta* (25 mg) with amber (37.5 mg). It is evident from previous studies that ambrex possesses hepatoprotective and antiulcerogenic properties.^[12,13] In the present investigation, we determined the influence of ambrex on oxidative stress in diet induced hyperlipidemic rats. In addition, we report that ambrex indeed suppresses the differentiation of 3T3-L1 preadipocytes via inhibition of the expression of master regulators of adipogenesis.

MATERIALS AND METHODS

Materials

Ambrex in capsule form was obtained from Care and Cure Herbs Ltd., Chennai. The assay kits for triglycerides and cholesterol (Merck make) were purchased from VKM Scientific Products, Chennai. Ambrex was dissolved in carboxymethylcellulose (CMC) and stored as stock solution and diluted with growth medium for *in vitro* studies. 3T3-L1 mouse fibroblasts (preadipocyte) were procured from National Centre for Cell Science (Pune, India). Cholesterol, egg yolk powder, sodium cholate, Dulbecco's minimum essential medium (DMEM), Fetal Bovine Serum (FBS), 3-isobutyl-1 methyl xanthine, dexamethasone, insulin, TRIZOL reagent were purchased from sigma, USA. All other chemicals used were of analytical grade.

Experimental protocol

A total of 24 male Wistar rats weighing about 130-150 g were maintained under standard husbandary conditions 25 \pm 5°C temperature, light/dark cycle with standard rat feed (Hindustan Lever Ltd.) and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC No. Biotech REC.001/10) and the experimental work was carried out as per Committee for the Purpose of Control and Supervision on Experiments on Animals guidelines. The experiment was divided into two periods, the former was the induction of hypercholesterolemia and the latter was treatment period (15 days). Induction of hypercholesterolemia (75% increase) continued for 30 days by feeding the rats with 10% egg yolk powder, 5% lard, 1% cholesterol and 0.5% cholic acid.^[14]

Then rats were divided into four groups of six animals each as follows:

- Group A: Normal basal diet receiving rats (Control group).
- Group B: Untreated hypercholesterolemic rats.
- Group C: Ambrex treated hypercholesterolemic rats (40 mg/kg body weight). Dosage was arrived from previous studies.^[13]
- Group D: Atorvastatin treated hypercholesterolemic rats (10 mg/kg body weight).

At the end of the experiment, the rats were fasted overnight and sacrificed by decapitation under mild anesthesia. Blood samples were collected and allowed to clot at room temperature and then centrifuged at 3,000 rpm for 30 min. The clear supernatant sera was removed and stored at -20° for subsequent analysis of total cholesterol, triglycerides, and antioxidant enzymes. Liver and heart tissues were dissected immediately and weighed. 0.5 g of liver and heart tissues were homogenized with 0.1 M potassium

phosphate buffer (pH 7.4) using a Teflon homogenizer. The homogenate was centrifuged and the supernatant was stored at -20° for the measurement of antioxidant markers such as GSH, SOD, CAT and lipid peroxides.

LPO of liver and heart homogenate was estimated by measuring the thiobarbituric acid reactive substances and was expressed in terms of malondialdehyde (MDA) content, according to the method of Mihara and Uchiyama.^[15] The MDA levels were estimated using 1,1,3,3, tetra ethoxypropane as the standard. Reduced GSH in blood was assayed by the method of Beutler *et al.*^[16] Reduced GSH in liver tissue was estimated as described by Ellman.^[17] SOD and CAT activities in serum and homogenates of liver and heart were determined by the method of Nishikim *et al.*^[18] and Aebi,^[19] respectively. Protein estimation was carried by the method of Lowry *et al.*^[20]

Cell culture

3T3-L1 fibroblasts were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin (100 IU, 100 mg/ml, respectively) at 37°C in 5% CO_2 . Cells were plated at a density of 2×10^5 cells in 60 mm petriplates and allowed to attach overnight. To induce differentiation, 2-day post-confluent 3T3-L1 preadipocytes were stimulated for 24 h by adding 51.8 mM 3-isobutyl, 1-methylxanthine, 0.25 μM dexamethasone, and 0.1%-(MDI) to the DMEM/10% FBS culture medium. After 48 h exposure to the differentiation medium, cells were maintained in DMEM, 10% FBS containing 1 μM insulin and for the subsequent 5-7 days MDI medium was replaced with DMEM and 10% FBS. The preadipocytes were exposed to ambrex (10 pg, 100 pg) 24 h prior to differentiation and throughout the study.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide MTT assay

Cytotoxicity assay using MTT was performed as per the protocol of Mosmann,^[21] with slight modification. In brief, 3T3-L1 cells were seeded in 24-well plate. Cells were incubated overnight at 37°C in a humidified atmosphere (5% CO_2). After reaching the confluence, 1 μl of Ambrex formulation at concentrations ranging from 10 pg to 1×10^7 pg were added to the wells in triplicates and incubated for 20 h. MTT was dissolved in phosphate buffered saline (PBS) at 5 mg/ml and filtered, 10 μl of MTT was added to each well and incubated for 4 h. After an incubation period, the medium was aspirated carefully from each well and 100 μl of Dimethylsulfoxide (DMSO) was added to all the wells. The color intensity was read at a test wavelength of 570 nm and a reference wavelength of 630 nm using Multiskan spectrum, USA. The inhibition (%) was expressed as the percentage of viable cell compared to control.

Oil Red O staining

After differentiation, 3T3-L1 pre-adipocytes were washed with PBS and then fixed for 1 h with pre-chilled 10% formaldehyde in PBS. Cells were stained with Oil Red O Solution (a mixture of three parts of 0.5% (w/v) Oil Red O in isopropanol and two parts of water) for 2 h at room temperature followed by washing twice with PBS, once with ethanol. Cells were then washed twice with sterile water and photographed using an inverted microscope.

Isolation of total RNA and reverse transcriptase-polymerase chain reaction analysis

After differentiation, total RNA was extracted from cultured cells using TRIZOL reagent (Sigma, USA). The isolated RNA was allowed to undergo reverse transcription and polymerization reaction to get cDNA using PCR master cyclor gradient. According to the protocol provided by the manufacturer, the RT-PCR was done using a RT-PCR kit (Genet Bio, Korea). 200 ng of RNA was reverse transcribed into cDNA. The sense primer sequence and antisense primer sequence for PPAR γ , SREBP, leptin, TNF- α , iNOS, TRX1 and TRX2 are summarized in Table 1. Beta-actin (sense: TCTACAATGAGCTGCGTGTG; antisense: GGTCAGGATCTTCATGAGGT) was used as an internal standard. The basic reaction conditions are as follows: DNA denaturation at 94°C for 5 min; PCR amplification; 94°C denaturation for 1 min, specific annealing temperature for 1 min, 72°C for 1 min (35 cycles), and final extension also at 72°C for 5 min. PCR products were loaded in 1.5% agarose gel, electrophoresed at 80V for 30 min. The specific bands were visualized with ethidium bromide staining. The intensities of the bands were measured using a gel documentation system.

Statistical analysis

All the data were expressed as mean \pm standard deviation. Statistical significance of data was analyzed by one-way analysis of variance (ANOVA) using Tukey's multiple comparison test using the Graph pad prism software package for windows (Version 5). $P < 0.05$ are considered as significant.

RESULTS

Effect of ambrex on oxidative stress

Table 2 details the serum lipid profile of various groups before and after the treatment protocol. It is evident that high fat diet group animals showed a significant rise in the serum total cholesterol and triglycerides levels. Administration of ambrex showed statistically significant decrease in serum total cholesterol (28.96%), triglycerides (37.94%) as compared to untreated hyperlipidemic rats. Figure 1 shows the activity of serum antioxidant enzymes SOD, CAT and GSH in various groups of animals. Decreased

activities of serum antioxidant enzymes were observed in hyperlipidemic rats when compared to normal rats. Ambrex treatment significantly elevated levels of SOD, CAT and GSH compared to untreated hyperlipidemic animals.

The consumption of high fat diet significantly augmented lipid peroxides level and reduced antioxidant enzymes SOD, CAT and GSH in the liver of rats [Figures 2 and 3] as compared to control group. Oral administration of ambrex decreased LPO and improved the antioxidant Enzymes activities significantly. The changes in the activities of the antioxidant enzymes, SOD, CAT and lipid peroxides levels in the heart tissues of the rats are summarized in Figures 3 and 4. The LPO of rats fed high fat diet was significantly higher than the control group. Levels of lipid peroxides decreased in heart ($P < 0.05$) when ambrex supplemented to the high fat diet fed rats in comparison to the control group [Figure 3]. There was a significant inhibition of the antioxidant status of heart during experimental hypercholesterolemia, specifically a decline in CAT and SOD activities [Figure 4]. Both SOD and CAT activities were found to be significantly enhanced in rats treated with ambrex ($P < 0.05$).

Effect of ambrex on 3T3-L1 adipocyte differentiation

It is a well-known fact that adipocyte differentiation involves an array of transcription factors. Intracellular fat accumulation can be assessed by Oil Red O staining which acts as a marker of adipogenesis. It is evident from the microscopic images, that ambrex attenuated lipid accretion in 3T3-L1 pre-adipocytes [Figure 5]. Ambrex treatment has significantly reduced cell differentiation compared to control cells. This indicates that ambrex blocks adipocyte differentiation effectively in 3T3-L1 cell line.

Effect of ambrex on mRNA expression of PPAR γ , SREBP, leptin, TNF- α , iNOS, TRX 1 and TRX 2

To examine the inhibitory mechanism of ambrex during adipocyte differentiation the expression levels of PPAR γ , SREBP, leptin, TNF- α , iNOS, TRX1 and TRX 2 were analyzed by RT-PCR to elucidate a detailed picture on lipogenesis, inflammation and oxidative stress associated with adipocyte differentiation. As shown in the Figure 6 ambrex significantly reduced the expression of mRNA of the adipogenesis related genes namely PPAR γ , SREBP and leptin, inflammatory-related genes namely TNF- α , iNOS, and increased the expression of oxidative stress related genes namely TRX1 and TRX2 in adipocytes.

DISCUSSION

In the present investigation both *in vivo* and *in vitro* studies were performed for better understanding of implications in diet induced hyperlipidemia and in 3T3-L1 cell line.

Table 1: Sequences of primers

Primer	Sequence (5'-3')
β -actin	5'-GACATGGAGAAAATCTGGCA-3' (sense) 5'-AATGTCACGCACGATTTC-3' (antisense)
Leptin	5'-CCCCATTCTGAGTTTGTCCA-3' (sense) 5'-GCTGAAGAACTAGGTGAGAG-3' (antisense)
PPAR γ	5'-GAGCTGACCCAATGGTTGCTG-3' (sense) 5'-GCTTCAATCGGATGGTTCTTC-3' (antisense)
SREBP	5'-ACCCCTGGTGGAGGGACCATCTTG-3' (sense) 5'-CTTTGCTTCAGTGCCACCACCAG GTCTTT-3' (antisense)
TNF- α	5'-ATGAGCACAGAAAGCATGATC-3' (sense) 5'-ACAGGCTTGTCACCTCGAATT-3' (antisense)
iNOS	5'-AATGGCAACATCAGGTCGGCCAT CACT-3' (sense) 5'-GCTGTGTGTCACAGAAGTCTCGAACTC-3' (antisense)
TRX 1	5'-CGTGGATGACTGCCAGGAT-3' (sense) 5'-GGTCGGCATGCATTTGACT-3' (antisense)
TRX 2	5'-TGTGGCCCTGCAAGATC-3' (sense) 5'-CCGTGCTGTTGGCTACCAT-3' (antisense)

PPAR γ : Peroxisome proliferator activated receptor gamma; SREBPs: Sterol regulatory binding proteins; TNF- α : Tumor necrosis factor; iNOS: Inducible nitric oxide synthase; TRX: Thioredoxin

Table 2: Effect of ambrex on serum total cholesterol, triglycerides in high fat diet fed male Wistar rats

Group	Total cholesterol (mg/dl)		Triglycerides (mg/dl)	
	Before treatment	After treatment	Before treatment	After treatment
A	57.00 \pm 2.11	59.17 \pm 2.82	64.67 \pm 4.44	66.33 \pm 4.44
B	89.67 \pm 2.89***	83.5 \pm 1.33***	86.67 \pm 2.56***	81.17 \pm 2.00**
C	86.83 \pm 2.89***	67.33 \pm 3.63##	73.33 \pm 2.48#	53.17 \pm 2.13####
D	83.67 \pm 3.21***	67.83 \pm 2.98##	71 \pm 1.06##	48 \pm 2.23#####

Values are expressed as mean \pm SEM for six animals in each group. P values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ statistically significant when compared with control group A. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ statistically significant when compared with high fat diet group B

Feeding of high fat diet markedly increased serum total cholesterol, triglycerides and administration of ambrex significantly decreased [Table 2] serum total cholesterol and triglycerides and thus substantiate the hypolipidemic potential of the herbal formulation, Ambrex. Reactive oxygen species are products of normal metabolism and interesting feature is its dual role as beneficial and deleterious variables. Imbalance between free radical generation and antioxidants results in oxidative stress. It is well-documented that free radical and insufficient antioxidant enzymes are the principal factors involved in the pathogenesis of hypercholesterolemia.^[22,23] Oxidative stress can damage lipids, proteins and DNA in cells and tissues leading to the loss of cell integrity and cell death. This scenario is the basis for the occurrence of several pathological conditions such as diabetes, cardiovascular disorders, autoimmune diseases. SOD and CAT are the

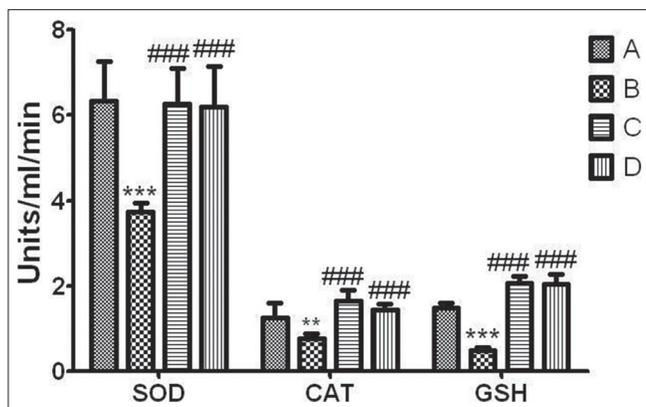


Figure 1: Effect of Ambrex on serum antioxidants of rats. SOD: Superoxide dismutase, CAT: Catalase, GSH: Reduced glutathione. Values are expressed as mean \pm SD for six animals in each group. *P* values: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 statistically significant when compared with control group A. #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 statistically significant when compared with high fat diet Group B

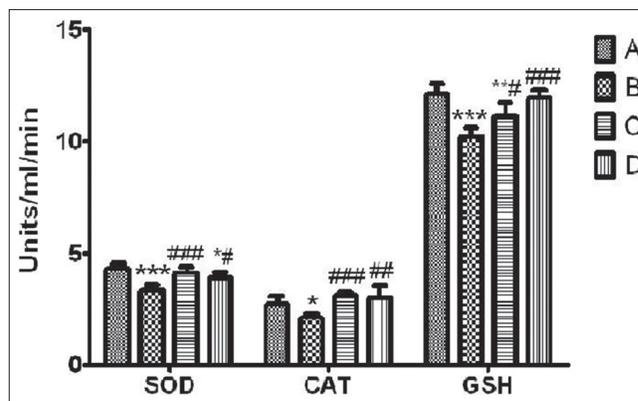


Figure 2: Effect of ambrex on hepatic antioxidants of rats. SOD: Superoxide dismutase, CAT: Catalase, GSH: Reduced glutathione. Values are expressed as mean \pm SD for six animals in each group. *P* values: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 statistically significant when compared with control group A. #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 statistically significant when compared with high fat diet Group B

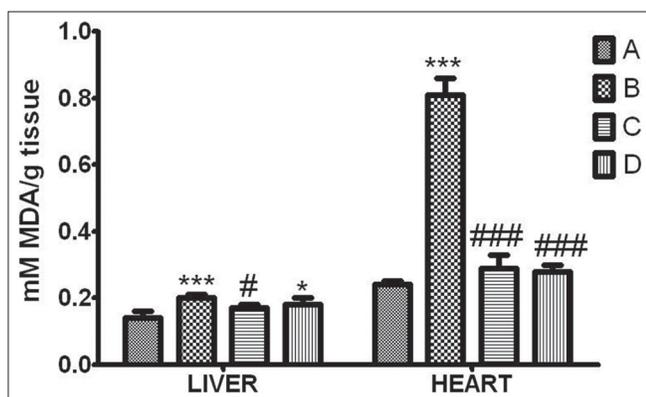


Figure 3: Lipid peroxidation of organ homogenates. LPO: Lipid peroxidation. Values are expressed as mean \pm SD for 6 animals in each group. *P* values: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 statistically significant when compared with control Group A. #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 statistically significant when compared with high fat diet Group B

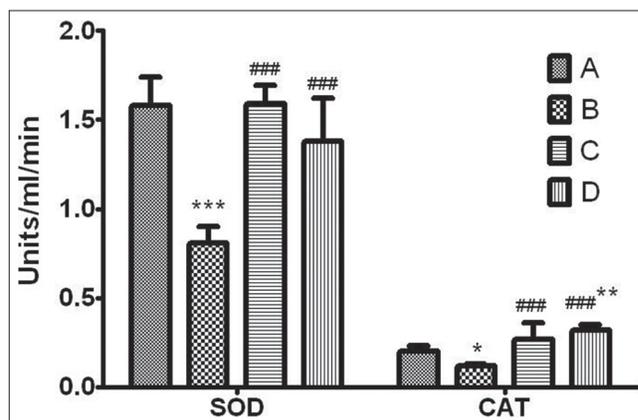


Figure 4: Effect of ambrex on heart antioxidants of rats. Values are expressed as mean \pm SD for six animals in each group. *P* values: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 statistically significant when compared with control Group A. #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 statistically significant when compared with high fat diet Group B

most potent enzymes involved in the elimination of oxidative stress. GSH acts as an endogenous scavenger of free radicals,^[24] and thus possess a defensive role against oxidative stress. SOD catalyses dismutation of superoxide into oxygen and hydrogenperoxide. CAT prevents free radical generation by catalyzing hydrogenperoxide into oxygen molecule and water. The experimental results reflected a reduced antioxidant activity and elevated LPO in the rats of high fat diet group (Group B) in the serum, liver, and heart tissues of the animals. The observed results are in concordance with findings of other researchers, which report that feeding a high fat diet to animals diminishes their antioxidant potential by increasing LPO and free radical production.^[25] The serum SOD, CAT and GSH activities were significantly inhibited in hyperlipidemic rats [Figure 1]. It is obvious from several studies that hyperlipidemia induced diet reduces the SOD, CAT, GPx

and GST activities and parallelly elevate the lipid peroxide levels, thereby leading to diminution of antioxidant defense system.^[26] Ambrex treatment has augmented the activities of SOD, CAT and GSH in the serum reversing the bad effects of hyperlipidemia. Alleviation in the activity of these enzymes attribute to elevation of free radicals that cause cell damage in these rats. The significant enhancement in the serum antioxidant enzyme activities of ambrex treated rats contribute a key role in the prevention of free radical generation.

Liver being the primary organ involved in cholesterol and lipid metabolism, transport and excretion, it is logical to assess the hepatic antioxidant status in hyperlipidemia condition. Hyperlipidemic rats showed marked reduction in the hepatic SOD, CAT and GSH activities as compared to the control. The decline was

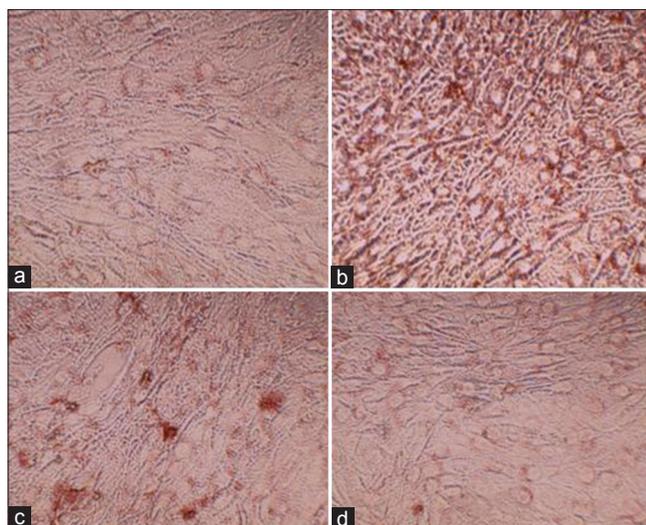


Figure 5: Oil Red O staining of triacylglycerol – containing vesicles in 8-day culture 3T3-L1 cells (a) Normal pre adipocytes (b) Differentiated adipocytes (c) Ambrex 10 pg and (d) Ambrex 100 pg

significantly restored ($P < 0.05$) in the presence of ambrex and atorvastatin when compared to high fat group. Ambrex has displayed protection against hepatic oxidative stress by enhancing antioxidant enzyme levels in the liver. MDA acts as an oxidant biomarker of LPO. In the present study, we found that high fat diet induced hyperlipidemic rats had elevated MDA levels. These results correspond to the report of Yalçın *et al.*^[27] Basic research details the role of LPO in oxidation of Low density lipoprotein (LDL) and its implications in cardiovascular disorders. However convincingly, LPO attenuated in ambrex treated animals which substantiate the antioxidant nature of it. The combined effect of decreased LPO and concurrent increase in antioxidant enzyme activities in the hepatic tissue would definitely lower the oxidative stress. In heart, high fat diet has been found to lower the SOD and CAT activities in rats as compared to normal rats. Work done by Kumar *et al.*^[28] also showed that high fat diet elevated serum lipid profile and oxidative damage to the heart tissue. Array of antioxidant enzymes, SOD, CAT and GSH-Px are involved in the protection mechanisms against oxidative stress. Supplementation of Ambrex in high fat diet fed rats resulted in augmentation of SOD and CAT activities when compared to untreated control. The LPO level markedly increased ($P < 0.05$) in the heart of Group B animals [Figure 3], which was significantly reduced by the treatment of ambrex and atorvastatin in Group C and Group D animals respectively. The beneficial activity of ambrex in the alleviation of oxidative stress may be due to the presence of active constituents like withanolides, catechin's in Ashwaganda together with flavonoids, polyphenols in ambrex. It is evident from previous studies that Ashwaganda enhances antioxidant enzyme activities.^[29]

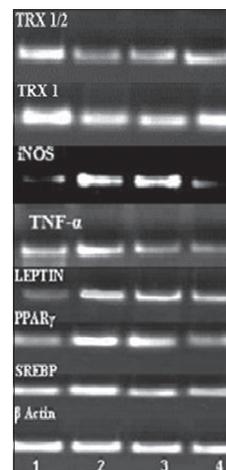


Figure 6: Reverse transcriptase-polymerase chain reaction for mRNA expression of leptin, peroxisome proliferator activated receptor gamma, sterol regulatory binding proteins, tumor necrosis factor- α , inducible nitricoxide synthase iNOS, thioredoxin (TRX 1 and TRX 2). Lane 1: Normal pre adipocytes, Lane 2: Differentiated adipocytes, Lane 3: Ambrex (10 pg), Lane 4: Ambrex (100 pg)

Adipocytes play a pivotal role in lipid homeostasis and energy balance.^[30] PPAR γ and SREBP are the major players in adipogenesis. Our *in vitro* experimental reports demonstrate that ambrex may be a potential drug against hyperlipidemia by repressing PPAR γ and SREBP regulated adipogenesis pathway. Leptin secretion is often correlated with augmented lipid accumulation,^[31] and lipogenesis.^[32] Ambrex may down regulate the expression of leptin, which may be the reason for decreased fat accumulation in adipocytes. An early instigator of inflammation and oxidative stress is adipose tissue. We report here that ambrex suppresses the mRNA expression of TNF- α , iNOS while augmenting the expression of TRX1 and TRX2 and thus prevents differentiation of 3T3-L1 pre-adipocytes and reduce oxidative stress.

Overall, the present study illustrates the potential antioxidant activity of ambrex in experimental hyperlipidemia. Our recent *in vitro* study,^[33] indicates that ambrex is rich in natural antioxidants. In this context, the observed effects of ambrex are pleiotropic and an outcome of many bioactive compounds. The present findings suggest that ambrex effectively blocks adipocyte differentiation in 3T3-L1 cultured cell line. Therefore, it may be a novel therapeutic agent for hyperlipidemia and related pathological disorders. Further research on these findings may provide a clear picture on exact molecular mechanism by which ambrex reduces adipocyte differentiation and oxidative stress.

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